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Bovine macrophages responses to the infection with virulent and attenuated *Leptospira interrogans* serovar Pomona

Ariel Nagel[a], Cristina Lourdes Vázquez[a], Julia ETULAIN[b], Federico Carlos Blanco[a], Maria José Gravisaco[a], Ricardo Martín Gómez[c], Karina Caimi[a,*]

[a] Institute of Agrobiotechnology and Molecular Biology (IABIMO), INTA-CONICET Buenos Aires, Argentina
[b] Laboratory of Experimental Thrombosis, Institute of Experimental Medicine, CONICET-National Academy of Medicine, Buenos Aires, Argentina
[c] Laboratory of Animal Viruses, Institute of Biotechnology and Molecular Biology, CCT-La Plata, CONICET-UNLP, Buenos Aires, Argentina

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ABSTRACT

Leptospirosis is a zoonosis, caused by pathogenic spirochetes of the genus *Leptospira.* Although cattle are usually the maintenance hosts of serovar Hardjo, Pomona is the most frequent serovar circulating in Argentina. The understanding of bovine innate immune response and the virulence of this serovar is important for future control measures. This work compares infection of bovine macrophages with the virulent *L. interrogans* sv Pomona strain AKRFB (P1) and its attenuated counterpart (P19). First, we confirmed attenuation in the hamster model. Mortality and lung hemorrhages occurred after P1 inoculation, while the survival rate was 100% in P19-infected animals. Cells infected with both strains showed statistically upregulated gene expression of pro-inflammatory cytokines, IL-1β, IL-6 and TNFα. The level of expression of anti-inflammatory cytokine IL-10 was statistically different between strains. Increased expression of IL-10 was observed only in P1-infected cells. For the first time, we describe macrophages extracellular traps induced by infection of bovine macrophages (bMETs) with both, the virulent and attenuated *Leptospira interrogans* Pomona strains. P1 was found higher internalized when the phagocytosis was inhibited, suggesting a cell entrance of this strain also by an independent-phagocytosis pathway. Furthermore, P1 was higher colocalized with acidic and late endosomal compartments compared with P19. This data emphasizes the importance to deepen in *Leptospira* bovine macrophages particular invasion mechanisms and, furthermore, underline the value of studying the main hosts.

1. Introduction

Leptospirosis has been described as the most widely distributed zoonosis in the world. This disease is caused by pathogenic spirochetes of the genus *Leptospira* that have been classified in several different species and further subdivided, based on surface antigens, into serovars (Haake and Levett, 2014). Leptospirosis is transmitted through contact with contaminated soil or water, and urine from infected mammals. Cattle are usually maintenance hosts of serovar Hardjo throughout the world (Ellis et al., 2000). Remarkably, in Argentina one of the most frequent serogroups in bovines is Pomona (Draghi et al., 2011; Petrakovsky et al., 2014), while in other countries it is mostly associated with swine (Bharti et al., 2003). Although the identification and characterization of this serovar is extremely important at epidemiological level, its pathogenicity or virulence attributes are still unknown.

The bovine immune system has also some similarities to humans with regard to pathogen recognition receptors (PRR). Cells in the innate immune system are activated following PRR engagement via a Toll-Like Receptor (TLR) (Baldwin and Telfer, 2015). Cattle are more similar to humans than the mouse model with regard to several aspects of immunophysiology, increasing its utility as a model to study the immune response to pathogen infection. Previous studies have highlighted differences in innate recognition of *Leptospira* between mouse and human cells (Li et al., 2010) and even greater differences are evident between mice and cattle innate immune recognition (Werling et al., 2009).

Neutrophils are the first-line of defense for the innate immune system. It has been shown that activated neutrophils undergo a form of cell death, called NETosis, in which nuclear DNA is released into the extracellular environment (Pilsczek et al., 2010). The resulting network of extracellular DNA and associated proteins (e.g., histones and granule constituents) are called neutrophil extracellular traps (NETs) and could serve as a physical barrier that prevents the further spread of pathogens (Pilsczek et al., 2010). Recently, Scharrig and colleagues have demonstrated, for the first time, NETs formation as result of *Leptospira*

* Corresponding author at: IABIMO, INTA-CONICET, Los Reseros y Nicolas Repetto s/n, 1712 Hurlingham, Buenos Aires, Argentina.
E-mail address: caimi.karina@inta.gob.ar (K. Caimi).

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stimulus using both human and murine models. Their results suggest that NETs formation is an important mechanism to prevent early leptospiral dissemination and also, to preclude further bacterial burden (Scharrig et al., 2015). In contrast, bovine neutrophils exhibited only modest or a slight activation in response to incubation with different *Leptospira* strains (Wilson-Welder et al., 2016).

Macrophages comprise a diverse group of cells that are found in all tissues and display diverse functions such as maintenance of homeostasis and immune surveillance (Wynn et al., 2013). These professional phagocytes play a key role in the innate ability to restrict the invading pathogens. Microorganisms internalized by phagocytic macrophages are sequestered in phagosomes, which are initially unable at killing and degrading pathogens. These phagosomes acquire their microbicidal properties through a complex maturation process that involves sequential fusion with endocytic organelles, which leads to the formation of phagolysosomes (Stuart and Ezekowitz, 2005). Chen and colleagues have demonstrated that leptospires are phagocytosed only by macrophages *in vitro* and *in vivo* and also they are resistant to phagocytosis by neutrophils (Chen et al., 2017). Furthermore, a study showed that *L. interrogans* survives and replicates within human macrophages but not within murine macrophages (Li et al., 2010). Another work suggests that pathogenic leptospires are able to survive, replicate and exit from mouse macrophages, enabling their eventual spread to target organs (Toma et al., 2011). In addition to classic macrophage phagocytic functions, bovine macrophages can also produce extracellular traps (ETs) in response to pathogen stimulus (Aulik et al., 2012).

On the other hand, one of the main concerns in the understanding of how hosts resist *Leptospira* infection has been the use of different *Leptospira* species, serovars, or strains that had undergone many *in vitro* passages in bacteriological media without first assessing infectivity (ID50) or lethality doses (LD50) before animal experimentation (Zuerner, 2015).

Because of the relevance of Pomona serovar for Public Health and livestock production in our country, we have used a comparative approach between the virulent and the attenuated counterpart of *L. interrogans* serovar Pomona strain AKRFB. The attenuation of Pomona strain gave us a tool to compare virulence and pathogenesis attributes under the same genetic background to deepen in different aspects of cattle innate immune response using an *ex vivo* model of primary bovine monocyte-derived macrophages (BMDMs).

2. Materials and methods

2.1. Ethics statement

The experimental animal work was carried out in accordance with the recommendations and approval of the Institutional Committee for the Care and Use of Experimental Animal (CICUAE) from the National Institute of Agricultural Technology (INTA) (Protocols No.44/2012 for hamsters and 02/2017 for bovine).

2.2. Maintenance and attenuation of L. interrogans serovar Pomona strain AKRFB

To characterize the virulence of a major serogroup circulating in our country, we have used *L. interrogans* sv. Pomona strain AKRFB. This strain was isolated from a fetal bovine kidney in 2007, during a leptospirosis outbreak that affected a dairy herd in Buenos Aires, Argentina. The strain was characterized by serological and molecular methods. It belonged to serogroup and serovar Pomona and its associated genotype in Argentina (ST52) (Koval et al., 2007; Varni et al., 2016). We have performed the strain attenuation by weekly serial culture passages in EMJH liquid medium. The leptospires were grown at 28 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium with 10% of *Leptospira* enrichment medium. The strain was inoculated in pathogen-free Syrian Golden hamsters (3 weeks old) to ensure a virulent phenotype. Briefly, the animals (n = 3) were intraperitoneally (IP) inoculated with 1 mL of a Lethal Dose (LD_{50}) of 50 leptospires that had been determined in a previous study (Koval et al., 2007). Five days post infection the animals were euthanized and the lungs were removed and homogenized. Four dilutions of each homogenate were inoculated in EMJH semisolid medium supplemented with 5-fluorouracil. The semisolid culture was incubated at 28 °C, and was monitored for *Leptospira´s* growth using Bacterial Live/Dead Viability Kit (Molecular probes, Invitrogen, USA). Once growth occurred, 100 μL of this culture was used to inoculate 30 mL of sterile EMJH media and the culture was incubated at 28 °C. This culture was named P1 and 1 mL aliquots were fractioned and kept until use in liquid N_2 tanks. The P1 isolate EMJH culture was subcultured by transferring 1 mL into 9 mL of sterile EMJH media (thus becoming P2), and incubated at 28 °C for 7 days. This process was repeated iteratively for 18 subcultures, and the final subculture was designated P19. Following the final subculture, all the passages were frozen for further use.

2.3. Attenuation assessment of L. interrogans serovar Pomona strain AKRFB

Previously to attenuation assessment, growth curves of P1 and P19 were performed (Supplementary Fig. 1A). Both cultures were stained 7 days after start-point of the growing curve (exponential phase) with BacLight LIVE/DEAD kit (Supplementary Fig. 1B). The attenuation was assessed using three-week-old male Syrian Golden hamsters. Before infection, leptospires were counting using optical density (OD). $OD_{420} = 0.35$ is equivalent to a 7.5×10^8 leptospires/ mL. Seven animals per group were IP inoculated with 1 mL of 1×10^8 leptospires of P1 and P19 resuspended in sterile phosphate buffered saline (PBS). The inoculum was taken after 7 days of growth according to the growth curve shown in Supplementary Fig. 1. Negative control animals were inoculated with 1 mL of PBS. The animals were monitored daily for characteristic signs of leptospirosis (i.e., prostration and jaundice) or survival. Animals that showed signs of acute infection between 5 and 7 days post infection (dpi) like decreased activity, anorexia, ruffled fur, etc., were euthanized immediately as this was established as critical end-point according to animal procedures. Surviving animals were euthanized after 21 days post inoculation. Animal lungs were recovered for histopathology studies and urine samples were taken for further leptospires growth in EMJH semisolid medium.

2.4. Isolation of bovine macrophages

Female heifers from Holando-Argentino breed were used in this study and they were selected from the experimental herd of INTA. The animals were determined sero-negative to Leptospirosis by microscopic agglutinating antibody test (MAT) if a titer lower than 1/100 was obtained for bovine serogroups panel recommended, including Pomona. A total of 200 mL of blood were taken in sterile conditions according to CICUAE protocols. Bovine peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by using Ficoll-Paque PLUS (GE) according to the manufacturer's instructions. Cells were seeded at 1×10^6 on 12-mm glass coverslips, 24-well plates and 1×10^7 on T25 flasks according to the experiment. Cells were cultivated in RPMI-1640 complete medium (Invitrogen, USA) supplemented with 10% of autologous plasma, 1% of 100U/ mL gentamycin and 100U/ mL streptomycin. Cells were incubated overnight at 37 °C under 5% CO_2. After pre-incubation, cells were thoroughly washed with sterile PBS pH 7.4 to remove non-adherent cells. Cells were continuously incubated for 5 days in RPMI-1640 supplemented medium to differentiate them into macrophages. As result of this treatment, bovine macrophages-derived from monocytes (BMDMs) were obtained from PBMCs. Cell viability was confirmed by Trypan blue exclusion assay. The identity of the cells was 93% using CD14+ staining by flow cytometry assay

Table 1
Primer sequences used in this study.

Genes	Oligonucleotide	Sequence (5´-3´)	Annealing temperature (°C)	Lenght (pb)
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH F	ATCTCTGCACCTTCTGCCGA	60	95
	GAPDH R	GCAGGAGGCATTGCTGACA		
Interleukin 1 β	IL-1β F	TGACCTGAGGAGCATCCTTT	60	137
	IL-1β R	CCAGCGATTTTTGCTCTCTG		
Interleukin 6	IL-6 F	TGCTTGATCAGAACCACTGC	60	146
	IL-6 R	GCGATCTTTTGCTTCAGGAT		
Interleukin 10	IL-10 F	GGAAGAGGTGATGCCACAGG	60	121
	IL-10 R	AGGGCAGAAAGCGATGACAG		
Interleukin 12	IL-12 F	TAGCCACGAATGAGAGTTGCC	60	76
	IL-12 R	TTTCCAGAAGCCAGACAATGC		
Major histocompatibility complex class II	MHCII F	ACAGTGACCATCTCCCCATC	60	103
	MHCII R	AACCACCGAACCTTGATCTG		
Toll-like receptor 2	TLR2 F	AACTCCATCCCCTCTGGTCT	60	106
	TLR2 R	TCAGGTTCACACACCTCTGC		
Tumor necrosis factor α	TNFα F	CCCCCAGAGGGAAGAGTCC	60	104
	TNFα R	GGGCTACCGGCTTGTTACTTG		
Lipoprotein *lip*L32	*lip*L32-45F	AAGCATTACCGCTTGTGGTG	52	242
	*lip*L32-286R	GAACTCCCATTTCAGCGATT		

(Supplementary Fig. 2).

2.5. BMDM infections

Freshly cultured of *L. interrogans* AKRFB-P1 and AKRFB-P19 were grown in EMJH liquid medium as describe above. Leptospires were harvested, washed and then were resuspended in antibiotic-free-RPMI-1640 medium. Bacterial concentration was adjusted to different multiplicities of infection (MOI) of 100, 50, 10 or 5 according to the sensitivity of each particular experiment. MOI of 100 was used for cytokines assay, 50 for the flow cytometry assays, 10 for bovine macrophages extracellular traps (bMETs) and 5 for intracellular trafficking assays (Li et al., 2010; Toma et al., 2011; Wilson-Welder et al., 2016). BMDMs were treated prior to flow cytometry and trafficking assays, with a final concentration of 20 µg/ mL of cytochalasin D, an actin filament disrupter that inhibits phagocytosis by macrophages (Liu et al., 2014), and incubated for 30 min in a CO_2 chamber at 37 °C. Infection was performed for 2 h at 37 °C and 5% CO_2 and then cells were washed twice with RPMI 1640. Three independent infections were performed for each assay.

2.6. Bovine macrophages extracellular traps

Prior to infection, leptospires were covalently stained with 5 (6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE, stock: 2 mM) (Sigma, Germany) in a final concentration of 10 µM. After the staining, leptospires were washed and suspended in 1 mL of PBS followed by dark incubation 30 min at room temperature. Leptospires were next washed with PBS until unbound colorant was eliminated and then used to infect BMDMs. *Confocal microscopy of bMETs*. Macrophages (5×10^5) were incubated for 3 h with labelled-P1 or P19 MOI of 10 at 37 °C in a humidified atmosphere with 5% CO_2 according to previous studies reported by Wilson-Welder and colleagues (2016). Cells were detached from tubes using a pipette tip and centrifuged using a Cytospin (ThermoFisher, USA) at 600 rpm for 6 min. Slides were fixed for 10 min with 4% paraformaldehyde (PF). Then, the slides were washed, blocked with PBS/ 1% SFB (serum fetal bovine, Internegocios) for 20 min at room temperature. The cells attached to the slides were finally incubated for 20 min with propidium iodide (PI) 2 mg/ mL (Sigma Aldrich, USA). Slides were washed and mounted using Aqua-Poly/ Mount coverslipping medium. Images were acquired using a FluoView FV1000 confocal microscope (Olympus, Japan) equipped with a Plapon 60X/NA1.42 objective, and then analyzed with Olympus FV10-ASW software. *DNA quantification*. Macrophages (5×10^5) were incubated as previously described and after incubation DNA released from macrophages during bMETs formation was digested with Micrococcal Nuclease (MNase) (500 mU/ mL) for 15 min. EDTA 5 mM was added to stop nuclease activity. Supernatants were collected, centrifuged and DNA was measured in the supernatants using PicoGreen (Invitrogen, USA) in a fluorometer (N = 6). The calibration curve was constructed using a thymus DNA of a known concentration. A one-way analysis of variance with Tukey's post hoc test was used for statistical analysis.

2.7. Isolation of RNA from infected BMDMs and cytokine quantification (RT-qPCR)

Macrophages were incubated for the 24 h with P1 or P19 using a MOI of 100 at 37 °C in a humidified atmosphere with 5% CO_2. After incubation time, the cells were lysed with 1 mL of chilled TRIzol (Invitrogen, USA) for RNA isolation. TRIzol was removed from the wells and the lysate was homogenized. Cellular RNA extractions were then performed according to the manufacturer's instructions for TRIzol reagent (Invitrogen, USA). RNA pellets were resuspended in 30 µL of RNase-free water. Total RNA quality and quantity was analyzed by spectrophotometry (NanoDrop 1000, ThermoFisher, USA) and electrophoresis on a 0.8% agarose gel. After treatment with Ambion™ DNase I (RNase-free) (ThermoFisher, USA), the DNA-free RNA (1 µg) was mixed with 50 ng of random primers (Invitrogen) in a 20 µL final volume and reverse transcribed to total cDNA with SuperScript II reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Approximately 25 ng of the cDNA was used as the template for each reverse transcriptase qPCR (RT-qPCR) analysis. RT-qPCRs reactions were performed on an Applied Biosystem Step One Plus instrument using Taq platinum, SYBR green I dye (Invitrogen, USA) and specific primers (Table 1) under standard cycling conditions. All reactions were performed in triplicates and qPCR curves were analyzed using LinReg (Ramakers et al., 2003). Fg software (Di Rienzo, 2002) was used for ratio calculation and statistical analysis (Pair Wise Fixed Reallocation Randomisation Test; 5000 permutations performed). To assess differences on gene expression in macrophages within groups, we used GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the reference gene and non-infected BMDMs as a calibrator (Blanco et al., 2009).

2.8. Flow cytometry assay

L. interrogans AKRFB-P1 and AKRFB-P19 was grown as previously described. Prior to infection leptospires were covalently stained with CFSE as previously described. Leptospires were next washed gently with PBS until unbound colorant was eliminated and then used to infect

BMDMs. BMDMs infections were performed seeding the cells in 12 well-plates and pre-treated with cytochalasin D as described. For this experiment, a MOI of 50 leptospires per cell was used. Cells infection was performed for 2 h at 37 °C and 5% CO_2 and then the cells were washed two times to eliminate the extracellular bacteria. After infection, 200 µL of Trypan blue 0.4% in PBS was added into each well for 5 min to quench fluorescence emitted from extracellular cell associated bacteria (Liu et al., 2014). BMDMs were scrapped, recovered in microtubes and then were centrifuged for 10 min at 2200 rpm. The cell pellets were fixed using 200 µL of 4% paraformaldehyde (PF) and then were incubated for 20 min at room temperature in the dark. Finally, another centrifugation step of 10 min at 2200 rpm was performed and the pellet was resuspended in PBS for flow cytometry analysis. Samples were analyzed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, USA) by gating on live bovine macrophages population based on forward and side scatter. For each measurement, data from 10,000 single cell events were collected. The macrophages became fluorescent due to association of CFSE-labelled leptospires. To obtain an estimated amount of internalized bacteria, the percentage of CFSE-positive cells was multiplied by the median fluorescence intensity of these cells obtaining the relative units (R.U.) of fluorescence. Data was analyzed by FlowJo software V.10 (FlowJo LLC, USA).

Statistical analyzes were performed using GraphPad prism 5.03 software (GraphPad Software, USA). Data were analyzed using two-way ANOVA and Bonferroni post-test for comparison among treatments (* statistically significant $p < 0.05$) and Wilcoxon match pair test for comparison between P1 and P19.

2.9. Indirect immunofluorescence

Bacteria preparation and BMDMs infections were performed as described for the flow cytometry assay but by seeding the cells in 24 well-plates with coverslips and using a MOI of 5 leptospires per cell in order to ensure individual leptospires counting.

Beads preparation. Polystyrene beads (3 µm; Krisker Biotech, Germany) were used as control of phagocytosis. Prior to infection, beads were coupled with autologous bovine serum. The coupling was performed using 0.1 M borate buffer (pH 8.5) at room temperature overnight on a rotating wheel. The beads were washed with BSA at 10 mg/ mL in borate buffer and finally were resuspended in PBS containing BSA at 10 mg/ mL and stored at 4 °C until use. *Internalization of polystyrene beads.* For internalization, coated beads were diluted 1:500 in complete RPMI supplemented with 10% autologous bovine serum and applied to bovine macrophages with a final concentration of ˜10 beads per cell. Beads were then incubated with BMDMs and, upon phagocytosis, the trafficking of bead-phagosomes (BPs) was analyzed using LAMP-3 and LysoTracker Red (Vázquez et al., 2017), and then analyzed by confocal microscopy.

Upon infection, cells were fixed with 4% PF solution in PBS for 20 min. The cells were permeabilized with 0.05% saponin in PBS containing 1% bovine serum albumin (BSA) for 15 min, followed by overnight incubation with the corresponding primary antibody diluted 1:50 in PBS. To label specific structures in bovine macrophages we used goat-anti-LAMP-3 antibody (Santa Cruz Biotechnology, USA). Secondary anti-goat antibodies conjugated to Alexa 488 (Jackson Immuno Research Labs, Inc., USA) were used diluted 1:500 in PBS for incubation time of 1.5 h. To detect acidic compartments, the dye LysoTracker Red (Invitrogen, USA) was used before fixation. The cells were incubated with 50 nM LysoTracker Red at 37 °C for 1 h. Finally, cells were mounted with mounting medium (Dako, Denmark) and analyzed by confocal microscopy.

2.10. Image analysis

Leica SP5 AOBS laser scanning confocal microscope (Leica Microsystems, Germany) from the Laboratorio Integral de Microscopía (LIM) of CICVyA, INTA was used. During image acquisition for fixed samples, a single focal plane was monitored in time (xyt scanning mode) using a 40X/1.25 HCX-PLAPO oil objective lens, and the same settings for laser powers, gain, and offset were maintained to compare the different experiments. The software of the microscope, LAS AF, allows saving the exact settings to acquire the images used in the experiments; therefore with every experiment the corresponding settings were loaded. An Argon laser (488 nm) and HeNe lasers (543 or 633 nm) were used in different combinations as required. Scanning was performed in sequential mode to eliminate signal bleed-through. Analyses of all the images were performed using ImageJ (U.S. National Institute of Health, Bethesda, Maryland, USA) and Fiji. Fiji is a distribution of ImageJ available at http://fiji.sc. Iterative versions of ImageJ used for this work are 1.41 m through 1.46a.

Leptospires internalization was monitored using the fluorescence of green CFSE per cell (n = 400), and LAMP-3 (recognized by a fluorophore (Cy5)-conjugated secondary antibody) or LysoTracker Red association with leptospires-compartments was analyzed in at least 250 compartments using Fiji software (U.S. National Institutes of Health, Bethesda, MD). The percentage of infected cells was estimated using 20 fields in each treatment at 40X amplification. The experiments were performed in duplicates in three independent experiments.

To measure the leptospires/ cell (i.e. the CFSE per cell, green channel), using the “line tool” from the toolbars of Fiji a single cell was outlined in the image. Then, the green channel was subjected to a pixel threshold and the “wand-tracing tool” from the Toolbars was used to select all the bacteria per cell. The “Analysed-Measure” function of Fiji was used to measure the fluorescence intensity of CSFE corresponding to a relative value of the amount of intracellular bacteria.

To measure the association of a LAMP-3 or Lysotracker Red with leptospires in the cell, the RGB image was split into individual channels. The red channel (bacteria) was subjected to a pixel threshold (using the same threshold for all the images). The “wand-tracing tool” was used to select all the bacteria per cell, and then the “Analysed-Measure” function was used to measure the fluorescence intensity of the marker of interest (corresponding to LAMP-3 or Lysotracker Red) associated to the bacterial phagosome by re-directing the measurements to the channel of interest in “Set Measurements” in Analyses function of Fiji.

Fluorescence intensity values were plotted and analysed using Microsoft Excel 2011 (Microsoft, USA) and GraphPad Prism 5 (GraphPad Software Inc., USA). Statistical analysis was performed using *t*-test.

3. Results

3.1. Attenuation of L. interrogans sv Pomona strain AKRFB

To insure that the loss in virulence of P19 was not due to a growth defect, we first performed a growth curves in EMJH medium of both strains and posterior staining with BacLight LIVE/DEAD kit (Supplementary Fig. 1).

The evaluation of the attenuated phenotype was performed in hamsters (n = 7) inoculated intraperitoneally with the passages P1 and P19. PBS 1X was used as control of the experiment. The LD50 was previously determined to be 50 leptospires (Koval et al., 2007). The P1 isolate was serially passaged in liquid medium for ~380 generations (18 weeks) to become P19.

Infections with virulent strains are associated with systemic infection and usually with severe acute disease in susceptible animals such as hamsters. Fig. 1A shows the mortality rate of hamsters inoculated with the different passages. All animals inoculated with P1 showed signs of acute infection between 5 and 7 days post infection (dpi) like decreased activity, anorexia, ruffled fur, etc., and were euthanized immediately as the critical end-points in the protocol for hamsters indicates. In contrast, 100% of animals inoculated with P19 showed no alteration in behavior, aspect or appetite; every animal survived and

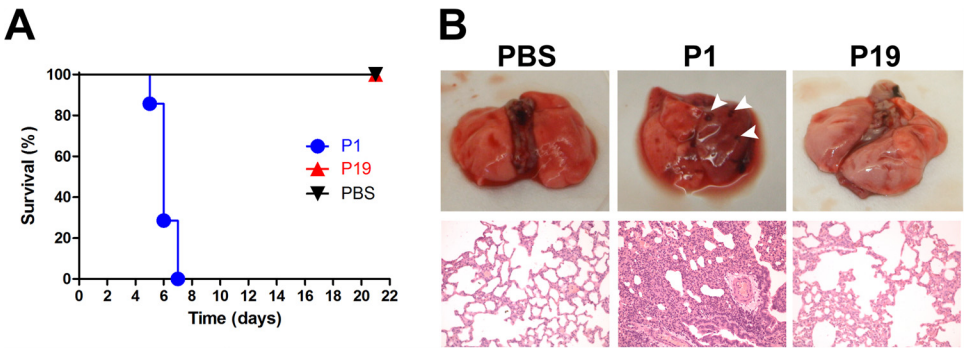

Fig. 1. Attenuation of *Leptospira interrogans* serovar Pomona AKRFB. Virulence was assessed in hamsters (n = 7) intraperitoneally inoculated with 1 mL of 1×10^8 bacteria per animal. **A.** Mortality rates. Survival percentage of treatments: P1 (blue), P19 (red) and PBS negative control (black). **B.** Pulmonary macroscopic (upper panel) and lung histopathology (lower panel) showing intra-alveolar hemorrhages with inflammatory infiltrates in lungs of P1-inoculated animals. The white arrowheads indicate hemorrhagic *foci* (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

was finally euthanized 21 dpi. The lungs from animals inoculated with P1 showed disseminated hemorrhagic focus (upper panel, Fig. 1B) and marked intra-alveolar hemorrhages with inflammatory infiltrates (lower panels, Fig. 1B) compared with lungs from control animals or from those inoculated with P19. The lungs of the control and the P19-inoculated animals show no signs of inflammatory infiltration. These results indicate that the strain P1 is virulent in hamsters, whereas P19 strain is attenuated in this model. Leptospires from urine samples of animals infected with both strains were recovered in EMJH semisolid medium indicating the systemic propagation of both strains (Data not shown).

3.2. Induction of immunomodulators in BMDMs

Macrophages can modulate local inflammation by production of pro-inflammatory cytokines and expression of different immunomodulators. To determine the innate immune profile established by bovine macrophages, we compared the gene expression level of different cytokines and immunomodulators after infection with P1 and P19. Bovine macrophages were infected and 24 h post infection, cells were lysed with Trizol and total RNA was isolated. Cytokines and immunomodulators gene expression for each strain was compared to uninfected cells and was normalized to housekeeping gene GADPH by RT-qPCR. Fig. 2 shows the level of gene expression of cytokines IL-1β, IL-6, IL-10, IL-12 and Tumor Necrosis Factor α (TNFα); and the immunomodulators Toll-Like Receptor 2 (TLR2) and Major Histocompatibility Complex class II (MHC II). The gene expression levels of IL-1β (p-value $_{P1} = 0.0013$, p-value $_{P19} = 0.0019$), IL-6 (p-value $_{P1} = 0.021$, p-value $_{P19} = 0.042$), IL-10 (p-value $_{P1\text{-}P19} = 0.005$, p-value $_{P1} = 0.0016$), and TNFα (p-value $_{P1} = 0.038$, p-value $_{P19} = 0.043$), were statistically higher in cells infected with both strains than those uninfected. Gene expression of IL-12, TLR2 and MHC II showed no differences between the cells infected with P1 or P19 and the uninfected controls. Interestingly, IL-10 showed statistically significant differences of expression between both strains, P1-infected cells induced higher level of expression of IL-10 than the cells infected with P19. As significant overproduction of IL-1 β and TNFα was observed for both strains, we determined the IL-10/TNFα and IL-10/IL-1β ratios of gene expression values. Both ratios were higher in P1-infected cells (IL-10/IL-1β: 0.061; IL-10/TNFα: 0.643) than in P19-infected cells (IL-10/IL-1β: 0.012.; IL-10/TNFα: 0.181) (Fig. 2, inset).

3.3. Formation of bovine macrophages extracellular traps (bMETs)

A previous work has demonstrated that bovine macrophages can produce extracellular traps (ETs) in response to pathogen stimulus (Aulik et al., 2012). To determine if bovine cells were capable of producing bovine macrophages extracellular traps (bMETs), we infected bovine macrophages with P1 and P19 that had been previously labelled with 5 (6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE). The generation of bMETs was evaluated using confocal microscopy.

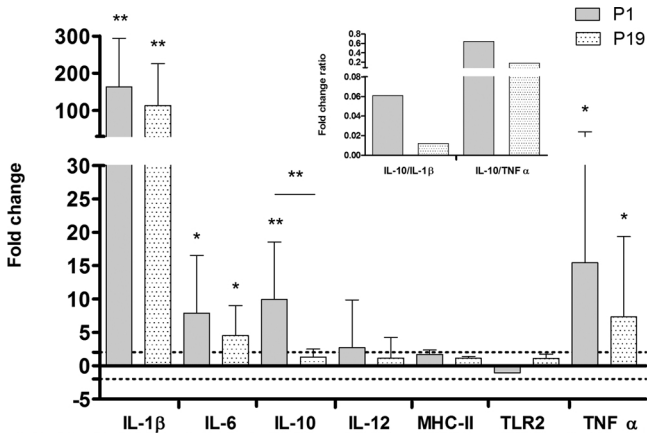

Fig. 2. Relative gene expression of different immunomodulators in bovine macrophages infected with P1 (grey) and P19 (black dots). Gene expression of IL-1β, IL-6, IL-10, IL-12, MHCII, TLR2 and TNFα was calculated using the $2^{-\Delta\Delta Ct}$ method with E correction. GAPDH mRNA expression was used as the reference gene and the uninfected control as the calibrator. P-values alone indicate statistical significance between infected cells and uninfected control and p-values with the bottom line (_) indicates statistical significance between P1 and P19. *p < 0.05, **p < 0.01. Average of three independent experiments calculated with standard deviation (SD). (IL-1β: p-value $_{P1} = 0.0013$, p-value $_{P19} = 0.0019$; IL-6: p-value $_{P1} = 0.021$, p-value $_{P19} = 0.042$; IL-10: p-value $_{P1\text{-}P19} = 0.005$, p-value $_{P1} = 0.0016$; TNFα: p-value $_{P1} = 0.038$, p-value $_{P19} = 0.043$; 5000 permutations performed). Inset shows the IL-10/IL-1β and IL-10/TNFα ratios.

Infected cells were labelled with propidium iodide (PI) and then the release of DNA to the extracellular medium was quantified. Fig. 3A shows confocal microscopy images confirming the presence of a DNA fibrils net released to the extracellular medium after infection with P1 (middle panels) and P19 (lower panels). The presence of leptospires associated to the DNA fibrils net was observed for cells infected with both strains. Quantification of released DNA rendered similar values for both strains compared with the non-infected cells that produced basal levels of DNA (Fig. 3B). In conclusion, we showed that both, P1 and P19 equally induced the formation of bMETs.

3.4. Quantification and association of leptospires with intracellular compartments

Phagocytosis plays a key role in the innate ability to restrict the spread of infectious diseases. Previous studies have demonstrated that at 1 h post infection a large number of leptospires are internalized (Toma et al., 2011). To deepen in the internalization process of leptospires, we infected BMDMs pre-treated or not with cytochalasin D, an inhibitor of actin polymerization and subsequently phagocytosis, with both P1 and P19 CFSE-labelled strains. After 2 h post infection cells were analyzed by flow cytometry (Fig. 4A). The fluorescence associated

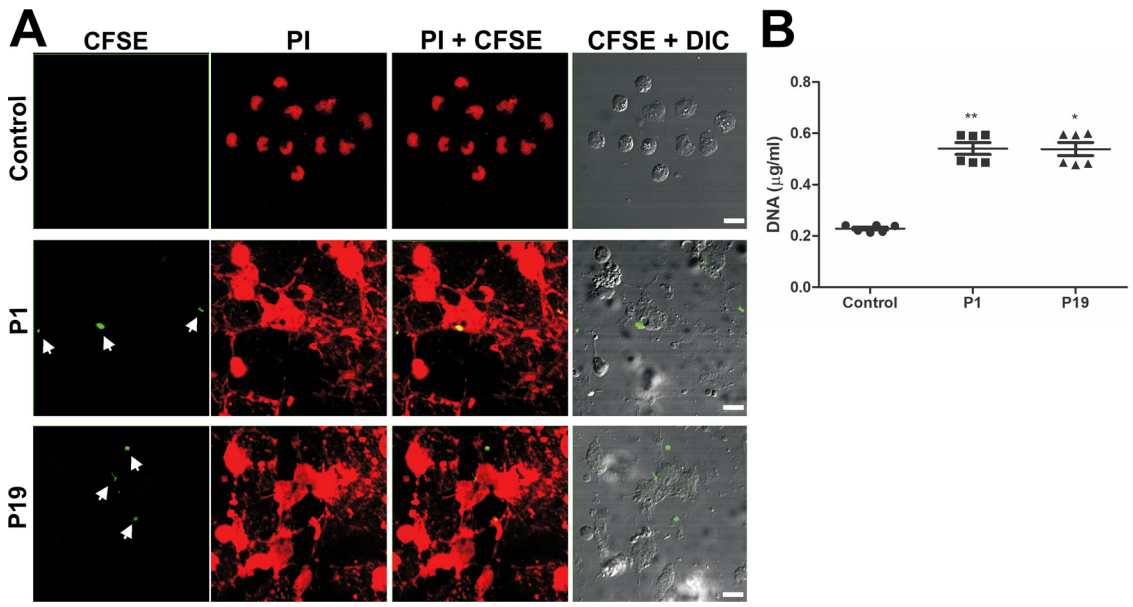

Fig. 3. Induction of bovine macrophage extracellular traps (bMETs). **A.** Confocal microscopy (60X) of BMDMs infected by CFSE labelled-P1 (middle panels) and P19 (lower panels) shows the induction of DNA fibrils net stained with propidium iodide (PI). The white arrows show labeled-leptospires. The upper panels show negative control of non- infected cells. Scale bar = 20 µm. **B.** Quantification of released DNA fibrils (µg/ mL) (N = 6). DIC: Differential interference contrast. Statistical analysis: one-way analysis of variance with Tukey's post hoc test, *$p < 0.05$, **$p < 0.01$ vs control.

to cells infected with P1 was higher than fluorescence in cells infected with P19 (square dots, Fig.4A). Similar results were obtained regarding the difference between fluorescence associated to cells infected with P1 or P19 when cells were pre-treated with cytochalasin-D (horizontal lines, Fig. 4A). This difference remained when cells were treated post-infection with Trypan blue, which quenches extracellular fluorescence. The cell-associated fluorescence decreased in P1 and P19 but the difference between them was still higher in P1 (vertical lines, Fig. 4A). Finally, the cells subjected to both treatments displayed also twice higher fluorescence when infected with P1 compared to P19. Even though no statistics difference was evident, these results demonstrate that P1 was more efficiently internalized than P19. Cytochalisin D does not impair internalization of both strains. These results suggest a possible phagocytic-independent internalization mechanism observed in the virulent strain that is less efficient in the attenuated strain.

With the aim to further understand the internalization process we quantified the interaction of leptospires with bovine macrophages. Therefore, we studied the internalization and the intracellular traffic of P1 and P19 after 2 h post infection using immunofluorescence staining against endosomal and lysosomal markers. The cells were also pre-treated with cytochalasin D as previously described. MOI of 5 was selected in order to be able of quantify individual leptospires inside the cells.

In parallel, to assess phagocytosis inhibition of cells pre-treated with cytochalasin D, we used polystyrene beads coated with autologous bovine serum. The trafficking of beads was analyzed by confocal microscopy using the endocytic marker lysosomal-associated membrane protein 3 (LAMP-3) and LysoTracker Red for acidic compartments (Vázquez et al., 2017). Bovine macrophages phagocytized beads when cytochalasin D was absent and the beads localized with both acidic (LysoTracker Red) and late endosomal compartments (LAMP-3, in green) (upper panels, Supplementary Fig. 3). The absence of phagocytized beads was evident after treatment with cytochalasin D (lower panels, Supplementary Fig. 3), which indicates an efficient inhibition of the phagocytic pathway in bovine macrophages.

Fig. 4B shows confocal microscopy images of cells infected with P1 (upper panels) and P19 (lower panels). P1 associated with intracellular LysoTracker Red (left upper panel), LAMP-3 (middle upper panel) and colocalization with both intracellular markers (right upper panel). Images show that after 2 h post infection the colocalization with the different markers was lower for the attenuated P19 strain than for P1. The percentage of cells infected with P1 was statistically higher compared to P19, independently of phagocytosis inhibition as observed previously by flow cytometry. Cytochalasin D treatment significantly reduced by 25% the number of P1-infected cells but this difference was not statistically significant for P19 (Fig. 4C). The quantification of the stained leptospires (CFSE) per cell (Fig. 4D) allowed us to confirm that intracellular P1 was statistically higher than P19 regardless the treatment applied. Indeed, intracellular P1 was still statistically higher than P19, when phagocytosis was inhibited. Thus, P1 could be entering the cells by phagocytosis but also by an independent-phagocytic pathway.

Additionally, we quantified the association of LysoTracker Red or LAMP-3 with the *Leptospira* compartments observed in Fig. 4B. Association of LysoTracker Red to compartment containing P1 was statistically higher than for the compartment containing P19 regardless the pre-treatment or not with cytochalasin D (Fig. 4E). Fig. 4F shows that association of LAMP-3 with compartment containing P1 strain was statistically higher than that for the compartment with attenuated P19 in absence of cytochalasin D. However, this difference was not observed when cytochalasin D was applied. The virulent P1 strain showed higher levels of internalization than the attenuated strain, even though both strains were able to enter the cells. P1 internalization occurred by means of a phagocytic-dependent but also by an independent pathway. In addition, the association of P1 with late endosomal compartments indicates that this strain undergoes a canonical traffic pathway within the time of infection (2 h). Moreover, its association with acidic compartments (LysoTracker Red) suggests that, this strain can survive the acidic environment.

4. Discussion

Cattle are becoming an important object of study because it represents a key host for many infectious and zoonotic diseases like leptospirosis. Bovine act as reservoir hosts of *Leptospira*, demonstrating chronic infection, with colonization limited to a few tissues (reproductive and urinary tracts) and intermittently shed *Leptospira*, infecting herd-mates, humans and other animals (Wilson-Welder et al., 2016). Studies to understand bovine innate immune response have

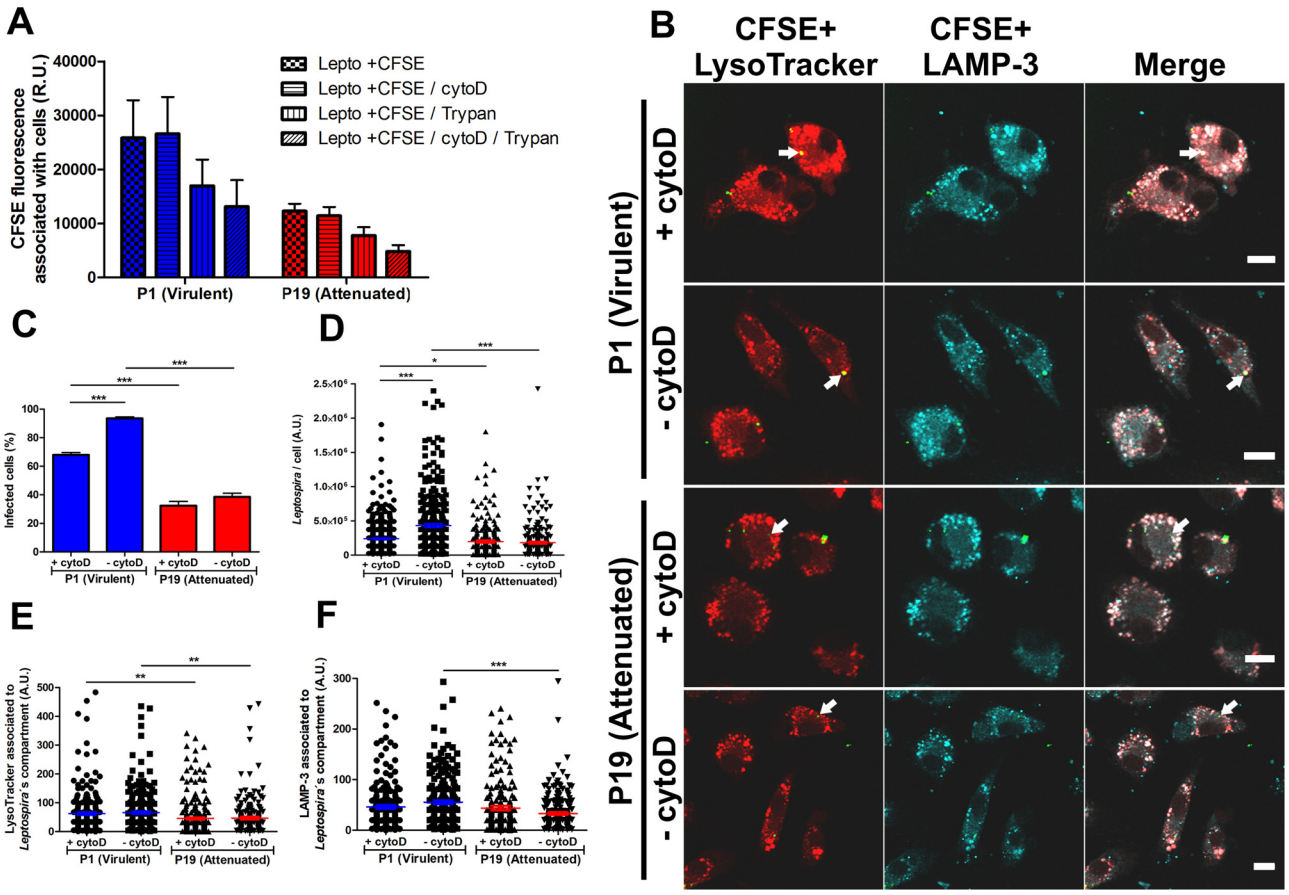

Fig. 4. Quantification and association of leptospires with intracellular compartments. **A.** Quantification of intracellular leptospires. Fluorescence of CFSE-labeled leptospires associated to BMDMs (R.U.) in different experimental conditions between P1 (blue) and P19 (red) was studied by flow cytometry. CFSE leptospires (square dots), CFSE -leptospires and cells pre-treated with cytochalasin D (horizontal lines), CFSE-leptospires and cells treated with Trypan blue (vertical lines) and CFSE-leptospires and cells pre-treated with cytochalasin D and Trypan blue (diagonal lines). Cyto D: Cytochalasin D. R.U.: Relative Units of fluorescence. The experiments were performed in four independent assays. Figure shows mean values with standard error (SEMs). Statistical analysis: Wilcoxon matched pair test, non-significant. **B.** Confocal microscopy representative images of cells infected with P1 (upper panels) and P19 (lower panels). Leptospires were stained with CFSE (in green); LysoTracker Red was used to stain acidic compartments and LAMP-3 (in cyan) for late endosomes. Intracellular leptospires are indicated with white arrows. The right panels show merge of CFSE, LAMP-3 and LysoTracker Red. Scale bar = 10 µm. **C.** Percentage of infected cells with P1 (blue) and P19 (red). Percentage was estimated using 20 fields (40X) in every treatment. **D.** Fluorescence intensity of stained leptospires (CFSE) per cell (n = 400). **E.** Fluorescence intensity of LysoTracker associated to *Leptospira´s* compartment per compartment (n = 250). **F.** Fluorescence intensity of LAMP-3 associated to *Leptospira´s* compartment per compartment (n = 250). Cyto D: Cytochalasin D. A.U.: Arbitrary Units of fluorescence. The experiments were performed in duplicates in three independent assays. Statistical analysis: t test, *p < 0.05, **p < 0.01, ***p < 0.001 with standard errors of the means (SEMs) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

lagged behind human or other animal models.

Bovine leptospirosis worldwide is due mainly to the infection with serovar Hardjo. Unlike what happens in other livestock producing countries, in Argentina Pomona is the main serovar circulating in cattle (Petrakovsky et al., 2014). In this work, we studied different aspects of innate immune response elicited upon infection of BMDMs with a virulent and attenuated variants derived from *L. interrogans* strain AKRFB that belongs to Pomona serogroup.

Up to now, many studies have compared *Leptospira* virulence, by using pathogenic or saprophytic strains from very different origins or genetic characteristics, without assessing the infectivity dose (Zuerner, 2015). Although several groups have previously reported low and high passages strains (Ristow et al., 2008; Lehmann et al., 2013), those studies pointed to different objectives. As result of 19 *in vitro* culture passages, the strain AKRFB of *L. interrogans* sv Pomona was attenuated. The attenuation was confirmed upon infection of hamsters, the acute model of disease that allowed us to assess the lethality dose (LD50). Although the systemic propagation was observed for both strains, the differential animal survival rate observed after infection with both passages were supported by histopathological examination that showed typical lesions found in both natural and experimental leptospirosis (Silva et al., 2008). Attenuation of Pomona strain gave us a tool to compare virulence and pathogenesis attributes using the same genetic background.

Macrophages play a key role in the innate ability to restrict the invading pathogens secreting a variety of inflammatory mediators that activate anti-microbial defense mechanisms. Although, these inflammatory macrophages are initially beneficial because they facilitate the clearance of invading organisms, they also trigger substantial collateral tissue damage because of the toxicity of reactive oxygen and nitrogen species. To counteract this inflammatory response, macrophages undergo apoptosis or switch into an anti-inflammatory or suppressive phenotype that dampens the pro-inflammatory response (Wynn et al., 2013). Cytokines are among the inflammatory mediators that orchestrate macrophages immune responses to infection. The differential regulation of cytokines expression was related to clinical outcomes in human leptospirosis (Matsui et al., 2017). Expression levels of cytokines including IL-10, IL-1β and TNF-α were previously evaluated as potential markers of leptospirosis severity in human, but little is known about the correlation of their expression level and disease

severity in bovine. In the present study, we observed increases in IL-1β, IL-6 and TNF-α cytokine gene expression after cells infection with both strains. Interestingly, bovine macrophages elicited higher levels of IL-10 gene expression when infected with P1 compared with P19. This suggests a preponderant role of this anti-inflammatory cytokine, that is also reflected by the higher IL-10/TNF-α and IL-10/IL-1β ratios in the virulent strain compared with the attenuated. The induction of IL-10 by the virulent strain could serve as a strategy to evade the pro-inflammatory macrophage responses in cattle. This mechanism is possibly related to resistance against leptospirosis in cattle as observed for other species (Matsui et al., 2012).

Unlike other spirochetes, *Leptospira* species possess LPS as part of their outer membrane. LPS is recognized by the pattern recognition receptors (PRRs), toll-like receptors TLR2 and TLR4, leading to pro-inflammatory cytokine, and chemokine responses (Nahori et al., 2005). Werts and colleagues have described that *Leptospira* LPS is recognized by TLR2 of human macrophages and by TLR2 and TLR4 in mice macrophages (Werts et al., 2001). Bovine macrophages express both TLR2 and TLR4, but TLR2 has a higher level of amino acid homology with the human TLR2. The differences among species in TLR structure that may impact in bacterial recognition and the little information about TLRs from bovine macrophages, lead us to test this receptor.

In addition to classic macrophage functions, recent investigation has demonstrated that macrophages are capable of producing extracellular traps (ETs) (Aulik et al., 2012). Our study is the first report on the induction of bovine METs upon infection with *Leptospira*. Although no significant difference was observed concerning DNA release to the extracellular medium between P1 and P19, both strains (virulent and attenuated) induced bMETs formation. Induction of bovine METs and NETs has already been described upon infection with other bacterial pathogens (Aulik et al., 2012; Hellenbrand et al., 2013). Leptospira induction of extracellular traps was reported in murine, human (Scharrig et al., 2015) and bovine neutrophils (Wilson-Welder et al., 2016), but for bovine neutrophils the induction of extracellular traps was similar for both pathogenic and saprophytic strains. Altogether, these results could indicate that cells from bovine origin may be unable to induce differential extracellular traps upon infection with strains of diverse virulence and this could act as a non-specific mechanism in bovine cells.

In neutrophils, the generation of NETs has been described as a mechanism to immobilize and kill microorganisms. In contrast and considering that monocytes and macrophages are powerful killing microbes than neutrophils, the extent of microbial killing attributed to METs could be no significant than that attributed to intracellular killing. However, recent evidences have proposed that METs may act synergistically with other components of host defense. Specifically, Halder and colleagues demonstrated that human monocyte release of METs is enhanced in the presence of human serum. During infection with *C. albicans* in media containing serum, they used immunofluorescence staining to establish that complement factors C3b and C5b-9 were deposited onto METs. They proposed that activated complement might add microbicidal activity and allow for the enhanced opsonization and phagocytosis of organisms within ETs during the resolution of inflammatory responses (Halder et al., 2016). In addition, Shen and colleagues showed that the antibiotic fosfomycin may boost MET release, potentially by increasing ROS production, and enhance the total extracellular killing of *S. aureus* by mouse peritoneal macrophages producing METs (Shen et al., 2016).

Regarding that, the release of bovine METs was similarly induced by P1 and P19, future studies are required to understand the possible local contribution of these extracellular structures as beneficial by killing microorganisms, or as detrimental by inducing a local cytotoxic effect in the bovine host.

As mentioned before, leptospires can locate in different intracellular compartments in macrophages from distinct hosts (Li et al., 2010; Toma et al., 2011). Flow cytometry and immunofluorescence followed by confocal microscopy assays allowed us to observed that the virulent strain internalized at higher levels than the attenuated strain regardless the pre-treatment with cytochalasin D in BMDMs. Liu and colleagues have shown a dramatically reduced uptake of leptospires when cytochalasin D was added to the cells (Liu et al., 2014). The higher intracellular localization of P1 (virulent) with inhibition of phagocytosis, allowed us to postulate a possible mechanism for entrance to the cell independent of phagocytosis. We have also observed a significantly increase in the association of acidic compartments (LysoTracker Red) with P1 compared with those containing the attenuated strain, thus suggesting a possible survival mechanism within 2 h post infection elicited by the virulent strain. The co-localization of the virulent strain with late endosomal compartments (LAMP-3) may also indicate that the virulent strain could also survive within those compartments 2 h post infection. Leptospires that have entered by a non-phagocytic way could be also later associated with acidic compartments (Fig. 4E) but more studies of intracellular traffic are needed at different time points after infection to fully subscribe this observation.

Although the molecular mechanisms of intracellular leptospires remain unknown because of the difficulties in genetic manipulation of this pathogen (Adler et al., 2011), here we provided a comparative approach using the same genetic background from a Pomona serogroup strain. To our knowledge, this is the first report that assesses different aspects of bovine macrophages innate immune response. This study allows us to further understand how *Leptospira* manipulates macrophages immune responses to enable the spreading of the infection.

Author contributions

Conceived and designed the experiments: A. N., J. E., M. J. G., R. M. G., K. C. Performed the experiments: A. N., C. L. V., J. E., F. C. B., M. J. G. Analyzed the data: A. N., C. L.V., F. C. B., M. J. G. Wrote the paper: R. M. G., K. C.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2019.04.033.

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